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The Late Endosomal Adaptor Molecule p14 (LAMTOR2) Regulates TGFβ1-Mediated Homeostasis of Langerhans Cells

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Abstract

Langerhans cells (LCs), a sub-population of dendritic cells (DCs) in the skin, participate in the regulation of immunity and peripheral tolerance. The adaptor molecule p14 is part of the late endosomal/lysosomal adaptor and mitogen-activated protein kinase and mammalian target of rapamycin (mTOR) activator/regulator (LAMTOR) complex, which mediates the activation of lysosome-associated extracellular signaling-regulated kinase (ERK) and the mTOR cascade. In previous work, we demonstrated that CD11c-specific deficiency of p14 disrupts LC homeostasis by affecting the LAMTOR-mediated ERK and mTOR signaling. In this study, we extended our analysis on p14 deficiency specifically in LCs. Langerin-specific ablation of p14 caused a complete loss of LCs, accompanied by an increased maturational phenotype of LCs. The absence of LCs in p14-deficient mice reduced contact hypersensitivity (CHS) responses to the contact sensitizer trinitrochlorobenzene. Analysis using bone marrow-derived DCs (BMDCs) revealed that p14 deficiency in DCs/LCs interfered with the LC-relevant transforming growth factor β1 (TGFβ1) pathway, by lowering TGFβ receptor II expression on BMDCs and LCs, as well as surface binding of TGFβ1 on BMDCs. We conclude that p14 deficiency affects TGFβ1 sensitivity of LCs, which is mandatory for their homeostasis and subsequently for their immunological function during CHS.

INTRODUCTION

Langerhans cells (LCs) are resident dendritic cells (DCs) in the epidermis of the skin and have been shown to participate in the induction of immunity, as well as peripheral tolerance

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CONFLICT OF INTEREST
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(Merad *et al.*, 2008; Romani *et al.*, 2010). To do so, they need to ingest, process, and finally present foreign antigen, as well as self-antigen, bound to major histocompatibility complex (MHC) molecules to T cells. A prerequisite for antigen presentation is an efficient, intracellular endosomal sorting machinery, which is controlled by several proteins including adaptor and scaffold molecules.

Among others, the adaptor molecule p14, also termed late endosomal/lysosomal adaptor and mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) activator/regulator complex 2 (LAMTOR2), regulates endosomal rearrangement, as well as other fundamental cellular processes such as growth factor signaling and proliferation in nonimmune but also immune cells (Teis *et al.*, 2006; Taub *et al.*, 2012). More importantly, deficiency of p14 is the molecular cause for the development of a hitherto unknown primary human immunodeficiency syndrome, which displays hallmarks reminiscent of diseases such as Griscelli and Hermansky–Pudlak syndrome (Klein *et al.*, 1994; Dell'Angelica *et al.*, 1999; Ménasché *et al.*, 2000; Jung *et al.*, 2006). Recent work demonstrated that p14 exerts its function by participating in the formation of the LAMTOR complex, which consists of five adaptor and scaffolding molecules: p18 (LAMTOR1), p14 (LAMTOR2), MP1 (LAMTOR3), HPXIP (LAMPTOR4), and C7orf59 (LAMTOR5). The LAMTOR complex represents the signaling platform important for the correct spatiotemporal activation of the extracellular signal–regulated kinase (ERK) MAPK, as well as the mTORC1 pathways at the stage of multivesicular bodies and lysosomes (Wunderlich *et al.*, 2001; Teis *et al.*, 2002; Nada *et al.*, 2009; Sancak *et al.*, 2010; Magee and Cygler, 2011; Bar-Peled *et al.*, 2012).

In previous work (Sparber *et al.*, 2014), we identified p14 as an important regulator for LC homeostasis, as its CD11c-specific deletion led to a virtually complete loss of LCs in p14-deficient mice. This was mainly owing to increased apoptosis and cell cycle arrest of LCs. At the molecular level, we determined that the p14-mediated disruption of the LAMTOR complex and the subsequently impaired ERK and mTOR signaling caused the loss of LCs. However, because of the pivotal function of p14 in regulating growth factor signaling, we sought to extend our previous findings and further investigated p14 deficiency in LCs with special focus on the LC-relevant transforming growth factor β1 (TGFβ1) signaling pathway.

RESULTS

Langerin-specific deletion of p14 leads to a loss of Langerin^{+} migratory DCs in the draining lymph nodes (LNs) of adult mice

Recently, we demonstrated that genetic deletion of the adaptor molecule p14 under the control of the *cd11c* promoter (CD11c-p14$_{del}$ mice) resulted in a complete loss of epidermal LCs. To further investigate p14 deficiency in LCs, we now specifically deleted p14 under the control of the *langerin/CD207* promoter by using Langerin-Cre mice (Zahner *et al.*, 2011) and analyzed the DC subsets in the skin-draining LNs of adult Langerin-p14$_{del}$ and littermate control mice. LNs of adult Langerin-p14$_{del}$ mice were of comparable size to those of wild-type controls, and we could not detect any significant differences in the percentages of total LN-resident CD11c^{high}MHC-class II^{int} DCs, nor in the various LN-resident CD11c^{+}CD4^{+}CD8^{neg}, CD11c^{+}CD4^{neg}CD8^{+}, and CD11c^{+} CD4^{neg}CD8^{neg}DC subsets (Figure 1a and b). This held true for mice of all ages analyzed, i.e., up to 2 months. In

addition, no difference in frequency of MHC-class $II^{low}CD11c^{+}PDCA1^{+}$ plasmacytoid DCs was observed (Figure 1a and c). In contrast, we noticed a significantly reduced percentage of the $CCR7^{+}$ migratory $CD11c^{int}$MHC-class II^{high}DC population in Langerin-$p14_{del}$ as compared with control mice owing to a marked reduction of the $Langerin^{+}$ skin-derived DCs (Figure 1a and d). Hence, our data demonstrate that *langerin*-specific ablation of p14 resulted in a specific loss of $Langerin^{+}$ migratory DCs in the skin-draining LNs of Langerin-$p14_{del}$ mice, which corroborates our previous observation concerning the loss of $Langerin^{+}$ migratory DCs after *CD11c*-specific deletion of p14 (Sparber *et al.*, 2014).

LCs are gradually lost in the skin of newborn Langerin-$p14_{del}$ mice

Similar to the analysis of CD11c-$p14_{del}$ mice (Sparber *et al.*, 2014), we analyzed the skin of newborn Langerin-$p14_{del}$ and control mice on defined time points after birth. In concordance with our previous data, we observed a gradual loss of epidermal LCs in Langerin-$p14_{del}$ mice. Intriguingly, we could not detect any significant difference in the percentages of LCs between Langerin-$p14_{del}$ and control mice during the first week after birth, suggesting normal population of the epidermis by LCs. However, p14-deficient LCs gradually declined approximately at 3 weeks of age (Figure 2a, b, and e). The delayed loss of LCs in the Langerin-$p14_{del}$ mice when compared with CD11c-$p14_{del}$ mice (Sparber *et al.*, 2014) can be explained by the different expression kinetics of CD11c and Langerin. Skin cell suspensions of newborn mice contained MHC-class II^{+} cells coexpressing CD11c; by day 3 after birth, most MHC-class II^{+} cells were positive for CD11c. In contrast, Langerin staining on MHC-class II^{+} cells was absent until day 7 after birth, when two-thirds of $CD11c^{+}$ cells coexpressed Langerin (Supplementary Figure S1 online). Reminiscent of CD11c-$p14_{del}$ animals, the loss of LCs in Langerin-$p14_{del}$ mice was accompanied by a more mature phenotype of the cells, as indicated by elevated expression of CD86 by $Langerin^{+}CD103^{-}$ LCs from whole skin cell suspensions (Figure 2c). At the same time, Langerin expression decreased on LCs (Figure 2c). In addition, in pure epidermal cell suspensions, we could confirm this enhanced maturation of p14-deficient LCs, as these cells showed increased MFI of CD86 and MHC-class II. As mentioned above, we noticed MHC-class $II^{+}Langerin^{low}$cells besides the MHC-class II^{+} $Langerin^{+}$ LC population (Figure 2f). Annexin-V revealed an increased staining, indicating that $Langerin^{low}$cells could represent apoptotic LCs that had partially lost Langerin and MHC-class II expression (Supplementary Figure S2 online). In contrast to LCs, we could not detect a reduction of $Langerin^{+}$ dermal DCs in Langerin-$p14_{del}$ mice (Figure 2d). This differs from our observation in the skin of CD11c-$p14_{del}$ mice, which displayed a significantly reduced frequency of $Langerin^{+}$ dermal DCs (Sparber *et al.*, 2014).

In summary, our data demonstrate that *langerin*-specific deletion of p14 leads to a gradual loss of LCs *in situ* starting 3 weeks after birth, which is accompanied by an increased phenotypic maturation status of the LCs and decreased expression of Langerin.

Absence of LCs in Langerin-$p14_{del}$ mice impairs contact hypersensitivity (CHS) response

As the role of LCs in CHS remains controversial (Noordegraaf *et al.*, 2010; Romani *et al.*, 2010), we assessed the immunological consequence of the loss of LCs in Langerin-$p14_{del}$ mice in the CHS response to the contact sensitizer trinitrochlorobenzene (Picrylchlorid,

TNCB). Langerin-p14$_{del}$ and control mice were sensitized with 1% (high dose) or 0.5% (low dose) TNCB on abdominal skin and 5 days later they were challenged with either 0.5% (high dose) or 0.25% (low dose) on the ear skin, respectively (Figure 3a). With both doses of TNCB, Langerin-p14$_{del}$ mice displayed a significantly reduced ear swelling reaction 24 hours after TNCB challenge as compared with controls (Figure 3b). Hence, our data demonstrate that the loss of LCs by *Langerin*-specific ablation of p14 reduces TNCB-mediated CHS responses, similar to animals with a selective LC deficiency due to disrupted TGFβ signaling (Zahner *et al.*, 2011).

CD11c-specific deletion of p14 impairs TGFβ1-mediated generation of LC-like bone marrow–derived DCs (LCL-BMDCs) and facilitates their enhanced maturation

Analysis of the molecular mechanism of p14 deficiency in CD11c-p14$_{del}$ BMDCs established that deletion of p14 leads to a partial disruption of the ERK and mTORC1 signaling pathway (Sparber *et al.*, 2014). The latter pathway has already been shown to affect LC homeostasis, as *CD11c*-specific deletion of the mTORC1-related regulatory-associated protein of mTOR (RAPTOR) molecule led to a loss of LCs in situ (Kellersch and Brocker, 2013). However, we observed an increased maturation of p14-deficient LCs, which was not apparent in RAPTOR-deficient mice (Kellersch and Brocker, 2013; Sparber *et al.*, 2014). As TGFβ1 is essential for the development and homeostasis of LCs, as well as the maintenance of the immature status of LCs in the epidermis of LCs in the skin (Borkowski *et al.*, 1997; Kaplan *et al.*, 2007; Kel *et al.*, 2010), we hypothesized that p14 deficiency might also affect TGFβ1 signaling in LCs. It is, however, very difficult to isolate sufficient amounts of highly purified LCs from the skin of newborn mice (Chang-Rodriguez *et al.*, 2005), particularly from the declining pool of LCs in Langerin-p14$_{del}$ mice. Therefore, we took advantage of BMDCs obtained from CD11c-p14$_{del}$ and control mice. Bone marrow precursors cultured in the presence of TGFβ1 give rise to a sub-population of Langerin^{+} LCL-BMDCs (LCL-BMDCs) (Valladeau *et al.*, 2002; Becker *et al.*, 2011). We first aimed to investigate the differentiation of these LCL-BMDCs, using this approach as a readout for the TGFβ1 sensitivity of p14-deficient BMDCs. It was therefore mandatory to delete p14 at the earliest possible time point during the bone marrow culture—that is, before the cells express Langerin. Therefore, we performed these experiments with CD11c-p14$_{del}$ mice, rather than with Langerin-p14$_{del}$ mice. We investigated the effect of p14 deletion on the differentiation of LCL-BMDCs in cultures supplemented with TGFβ1 for 8 days. Intriguingly, significantly less LCL-BMDCs could be generated from CD11c-p14$_{del}$ BM as compared with control cultures, suggesting impaired TGFβ1 signaling in p14-deficient BDMCs. Furthermore, CD11c-p14$_{del}$ LCL-BMDCs exhibited significantly elevated expression of the maturation marker CD86 in terms of both percentage of positive cells and mean fluorescence intensity (Figure 4a and b). Since a recent report identified bone morphogenetic protein (BMP)7 as an important factor for the differentiation of human LCs (Yasmin *et al.*, 2013), we tested its effect on the generation of LCL-BMDCs. In contrast to TGFβ1, the addition of BMP7 at various concentrations (50-200 ng ml^{-1}) throughout the BMDC culture could not induce differentiation of LCL-BMDCs in the murine system (data not shown). The addition of TGFβ1 to DC cultures impairs the upregulation of maturation markers such as MHC-class II, and CD86 by BMDCs (Strobl *et al.*, 1997; Yamaguchi *et al.*, 1997; Geissmann *et al.*, 1998; Caux *et al.*, 1999). We hypothesized that p14-deficient

BMDCs are less sensitive to TGFβ1 and thus more mature even in the presence of abundant maturation-dampening TGFβ1. Indeed, $CD11c^+$ BMDCs of CD11c-$p14_{del}$ mice, generated both in the presence and absence of TGFβ1 for 8 days, showed an increased expression of the maturation markers MHC-class II, CD86, and CD40 (Figure 4c and d). The inferiority of CD11c-$p14_{del}$ BMDCs to induce TGFβ1-mediated LCL-BMDCs, combined with increased expression of MHC-class II, CD86, and CD40 in cultures supplemented with TGFβ1 is reminiscent of the phenotype of Langerin-$p14_{del}$ LCs *in vivo*. These findings suggest that p14 deletion affects TGFβ1 responsiveness in $CD11c^+$ BMDCs/LCL-BMDCs, as well as LCs.

BMDCs generated from p14-deficient mice reveal decreased expression of TGFβRII and diminished surface binding of TGFβ1

Owing to the decreased sensitivity of p14-deficient BMDCs toward TGFβ1, we asked whether the surface expression of TGFβ1-binding receptors was altered in p14-deficient BMDCs. Indeed, FACS analysis of the TGFβRII subunit revealed a decreased expression level of this receptor on immature $CD11c^+CD86^-$ and mature $CD11c^+CD86^+$ p14-deficient BMDCs (Figure 5a). For direct analysis of the binding capacity of TGFβ1 to cell surface receptors, we incubated BMDCs on day 8 of culture with biotinylated TGFβ1 followed by a fluoresceinated avidin detection reagent. CD11c-$p14_{del}$ BMDCs bound significantly less TGFβ1 as compared with control cells, indicated by the reduced mean fluorescence intensity of TGFβ1 staining measured by flow cytometry. This held true for BMDC cultures in the absence and presence of additional TGFβ1 (Figure 5b and c). The reduced TGFβ1 binding of BMDCs from TGFβ1-containing cultures, as compared with nonsupplemented cultures, was presumably caused by saturation and/or internalization of TGFβ receptors. Microscopic analysis further emphasized our flow cytometry data, as p14-deficient BMDCs exhibited reduced fluorescence intensity when incubated with fluorescent TGFβ1 (Figure 5d).

Diminished TGFβRII expression on epidermal LCs from Langerin-$p14_{del}$ mice

For direct assessment of TGFβ surface receptor expression on LCs, we used LCs isolated from newborn skin of 21-day-old Langerin-$p14_{del}$ and control mice. Antibody staining of TGFβRII revealed significantly reduced expression on Langerin-$p14_{del}$ as compared with control LCs (Figure 6a and b). In the light of recent publications demonstrating that LC homeostasis is impaired by deficient IL-34 signaling (Greter *et al.*, 2012; Wang *et al.*, 2012), we analyzed the expression of the IL-34 receptor, i.e., the M-CSF receptor (CD115), on p14-deficient BMDCs and LCs (Supplementary Figure S3 online). However, we could not detect any differences in the expression of the M-CSF receptor between p14-deficient and control BMDCs/LCs (Supplementary Figure S3 online). In addition, we did not observe alterations in the expression of the GM-CSFR (CD116), a receptor that has also been implicated in the regulation of LC homeostasis and maturation (Burnham *et al.*, 2000) (Supplementary Figure S3 online). Thus, p14 deletion negatively regulates the expression of TGFβRII by LCs.

DISCUSSION

Several reports have demonstrated a pivotal role of the late endosomal adaptor molecule p14 in fundamental cellular processes such as cell proliferation, growth factor signaling, and

endosomal rearrangement in nonimmune but also immune cells (Teis *et al.*, 2006; Taub *et al.*, 2012). Analysis of the molecular mechanism underlying these processes revealed a protein complex called LAMTOR, which consists of five molecules: p18 (LAMTOR1), p14 (LAMTOR2), MP1 (LAMTOR3), HPXIP (LAMTOR4), and C7orf59 (LAMTOR5). Recruitment and assembly of the LAMTOR complex at the stage of multivesicular bodies and lysosomes represents a prerequisite for the subsequent, spatiotemporal activation of the ERK MAPK cascade (Wunderlich *et al.*, 2001; Teis *et al.*, 2002; Nada *et al.*, 2009), as well as the mTOR cascade (Sancak *et al.*, 2010; Bar-Peled *et al.*, 2012). In our previous work, we demonstrated that *CD11c*-mediated deficiency of p14 has a profound impact on the population of epidermal-resident LCs resulting in a complete loss of LCs in adult mice owing to apoptosis and cell cycle arrest. Moreover, we were able to establish that p14 deletion critically affects signaling of the LAMTOR complex through the ERK MAPK and mTOR cascade in DCs (Sparber *et al.*, 2014).

In this study, we extended our findings in the CD11c-p14$_{del}$ mice and investigated additional effects of p14 deficiency specifically in LCs. To this end, we deleted p14 under the control of the *langerin* promoter. Similar to CD11c-p14$_{del}$ mice, *langerin*-specific ablation of p14 results in a gradual loss of LCs starting approximately 3 weeks after birth. Importantly, we noticed divergent kinetics for the disappearance of LCs in CD11c-p14$_{del}$ and Langerin-p14$_{del}$ mice, with a delayed loss of LCs in the latter. A possible explanation for this discrepancy is the temporally distinctive activation of the *cd11c* and *langerin* promoters during neonatal LC development. The expression of CD11c is quickly induced on LCs in the epidermis of newborn mice between days 0 and 4 (Chorro *et al.*, 2009 and Supplementary Figure S1 online), indicating a very rapid activation of the CD11c promoter. In contrast, Langerin can be detected earliest by day 5 after birth in about half of the LCs, and only by days 7–10 most LCs express Langerin (Tripp *et al.*, 2004 and Supplementary Figure S1 online). In contrast to CD11c-p14$_{del}$ mice in which both Langerin-expressing skin DC subsets were reduced (Sparber *et al.*, 2014), deletion of p14 using Langerin-Cre mice affected only LCs but not Langerin^{+} dermal DCs. The p14 molecule interferes with many different cellular mechanisms in LCs, e.g., cell cycle arrest and apoptosis, and some of these effects may also affect Langerin^{+} dermal DCs upon p14 deletion. However, this was not investigated in further detail in this study and remains elusive. Interestingly, in the Langerin-p14$_{del}$ mice, the population of Langerin^{+} dermal DCs seems unaltered, which may be caused by the delayed activation of the langerin promoter and the faster turnover and repopulation kinetics of Langerin^{+} dermal DCs (Bursch *et al.*, 2007; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007) when compared with LCs (Noordegraaf *et al.*, 2010). In combination, this will lead to a postponed deletion of p14 under the *langerin* promoter and a fast replacement by new p14-sufficient dermal Langerin^{+} DCs, which together might conceal the p14-driven loss of this population observed in the skin of CD11c-p14$_{del}$ mice (Sparber *et al.*, 2014).

During the past decade, the functional role of LCs has been disputed because of the fact that the three mouse models allowing depletion of Langerin + dermal DCs and/or LCs (Bennett *et al.*, 2005; Kaplan *et al.*, 2005; Kissenpfennig *et al.*, 2005) yielded conflicting results, particularly in the CHS model (Romani *et al.*, 2010). Hence, we performed CHS assays with the contact sensitizer TNCB in adult Langerin-p14$_{del}$ mice completely lacking LCs and

observed a reduced CHS response. Although this is in contrast to a possible regulatory function of LCs in CHS (Kaplan *et al.*, 2005; Bobr *et al.*, 2012), our finding provides further evidence for a role of LCs in the efficient induction of CHS, which is in line with several reports suggesting that both LCs and Langerin + dermal DCs contribute to CHS induction (Bennett *et al.*, 2005, 2007; Bursch *et al.*, 2007; Edelson *et al.*, 2010; Honda *et al.*, 2010; Kel *et al.*, 2010; Noordegraaf *et al.*, 2010; Zahner *et al.*, 2011). Thus, in the case of Langerin-p14$_{del}$ mice, the attenuated CHS reaction may be due to the lack of LCs or, alternatively, to functional alterations of Langerin+ dermal DCs. However, we have not further explored the latter possibility in Langerin-p14$_{del}$ mice in this study.

In our prior work, we established that various signal transduction molecules involved in the mTORC1 pathway are significantly reduced in p14-deficient BMDCs (Sparber *et al.*, 2014). Here, we take this finding a step further and demonstrate that the differentiation of LCL-BMDCs is compromised upon deletion of p14. To a certain extent, this is reminiscent of previous work, showing that *CD11c*-specific deletion of the mTORC1-related molecule RAPTOR leads to the loss of LCs *in situ* (Kellersch and Brocker, 2013). However, comparison of the LC phenotype of RAPTOR-deficient and CD11c-/Langerin-p14$_{del}$ mice reveals several differences. Most prominently, the enhanced maturation of p14-deficient LCs is not observed in RAPTOR-deficient LCs. As TGFβ1 is essential for maintaining the LCs in an immature state in the skin (Kel *et al.*, 2010; Bobr *et al.*, 2012), we examined the effect of TGFβ1 on p14-deleted BMDCs. Moreover, the development of LCs is critically influenced by TGFβ1 (Borkowski *et al.*, 1996; Kaplan *et al.*, 2007), and LC-like cells can be generated in vitro in cultures supplemented with TGFβ1 (Strobl *et al.*, 1997; Yamaguchi *et al.*, 1997; Geissmann *et al.*, 1998; Caux *et al.*, 1999). Indeed, we observed an impaired differentiation of LCL-BMDCs from bone marrow of CD11c-p14$_{del}$ mice *in vitro*. This was accompanied by higher expression of MHC-class II, CD86, and CD40 indicative of enhanced maturation of CD11c-p14$_{del}$ as compared with control BMDCs even in the presence of exogenous TGFβ1. This finding suggests that p14 expression is essential for the responsiveness of BMDCs toward TGFβ1 that would keep these cells in an immature state. In fact, the increased maturation in p14-deleted BMDC mirrors the LC phenotype observed in Langerin-p14$_{del}$ mice *in situ*, as well as in mice with a LC-specific deletion of the TGFβ receptor 1 (ALK5) (Kel *et al.*, 2010).

Previous work already demonstrated that deletion of p14 led to an impaired endosomal sorting of the EGFR (Teis *et al.*, 2006), and thus it is conceivable that p14 deficiency might also affect other growth factor receptors such as TGFβ receptors. In line, we detected a reduced surface binding of TGFβ1 to p14-deficient BMDCs and less surface expression of TGFβRII on *ex vivo*-isolated LCs. However, to date, there is no causal link established between the adaptor molecule p14 and the TGFβ pathway. Whether p14 deletion in LCs directly affects TGFβ receptors by disrupting receptor shuttling processes, or whether the decrease of TGFβ receptors is rather an indirect response to the p14 deletion, remains elusive. Previous publications demonstrated that other receptors such as the IL-34-binding M-CSFR (Greter *et al.*, 2012; Wang *et al.*, 2012) and the GM-CSFR (Burnham *et al.*, 2000) are important for the homeostasis and maturation of LCs *in situ*. However, FACS analysis

did not reveal any differences in M-CSFR and GM-CSFR cell surface expression between p14-deficient and p14-sufficient BMDCs and LCs.

Taken together, ablation of p14 under control of the langerin promoter leads to a gradual but delayed loss of LCs as compared with CD11c-p14$_{del}$ mice owing to increased apoptosis and cell cycle arrest of LCs in the epidermis (Sparber *et al.*, 2014). The hyporesponsiveness to TGFβ1 as observed in CD11c-/Langerin-p14$_{del}$ mice might enable enhanced apoptosis, as TGFβ1 and apoptosis have been linked; e.g., TGFβ1 can inhibit apoptosis through the MAPK pathway (Park *et al.*, 2005, Ohtani *et al.*, 2009). The enhanced maturation of LCs can be ascribed to the insensitivity of p14-deficient cells to TGFβ1 present in the skin, most probably caused by reduced TGFβ receptor expression on the surface of LCs. Thus, the discovery that p14 deletion affects the TGFβ1 pathway, besides the already known defects in ERK and mTOR signaling, adds an important aspect to the molecular mechanisms that control LC homeostasis *in vivo*. Hence, the published study on CD11c-/Langerin-p14$_{del}$ mice (Sparber *et al.*, 2014) and the data presented here are complementary and the finding that the TGFβ1 pathway is disturbed in the p14-deficient cells represents an additional explanation as to how the maintenance of immature LCs, as well as their differentiation, are affected in these mice.

MATERIALS AND METHODS

Mice

The following mice were used: Langerin-Cre (Zahner *et al.*, 2011), CD11c-Cre (Caton *et al.*, 2007), and p14-flox mice (Teis *et al.*, 2006). Langerin-Cre or CD11c-Cre mice were crossed to p14-flox mice to generate LC- or DC-specific p14-deficient mice: *Langerin*-Cre/p14$^{flox/flox}$ and *CD11c*-Cre/p14$^{flox/flox}$(Langerin-p14$_{del}$/CD11c-p14$_{del}$). All mice were used at 6–8 weeks of age for experiments with adult mice or younger, as indicated in newborn skin analyses. Mice were bred at the animal facility of the Department of Dermatology and Venereology. All experimental protocols were approved by the Austrian Federal Ministry of Science and Research and performed according to institutional guidelines.

PCR genotyping

DNA was isolated from the tail of mice using Qiagen TailLysis Buffer (Qiagen, Venlo, The Netherlands). Appropriate primers (Microsynth, Balgach, Switzerland) and analysis of particular genetic loci were used, as described previously (Teis *et al.*, 2006), in order to identify CD11c-p14$_{del}$/Langerin-p14$_{del}$ mice.

Flow cytometry

All antibody-staining steps were performed for 15 minutes at 4 °C. Nonspecific FcR-mediated antibody staining was blocked by incubation with anti-CD16/32 Ab (2.4G2, prepared in-house from hybridoma supernatant). The following antibodies were used: anti-Langerin-Alexa 488 (929.F3, Dendritics, Lyon, France); anti-MHC-class II-FITC (553632), anti-CD86-PE (553692), anti-CD40-PE (55379), and anti-*CD103*-PE (557495) (all from BD Biosciences, Vienna, Austria); anti-*CD11c*-PE-Cy5.5 (15-0114-81), anti-MHC-class II-APC (17-5321-81), and anti-M-CSFR-APC (17-1152-82, all from eBioscience, San Diego, CA);

APC-coupled Life/Dead Cell stain kit (L10120, Life Technologies, Carlsbad, CA); and unconjugated anti-TGFβRII (sc-400) and anti-GM-CSFR alpha (sc-25472, both from Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody Alexa fluor488 goat anti-rabbit IgG was used to detect anti-TGFβRII and anti-GM-CSFR antibodies (A31628, Life Technologies). TGFβ-binding assays were carried out using the TGFβ1 Fluorokine Kit (NFTG0, R&D Systems, Minneapolis, MN) according to the manufacturer's guidelines. For Annexin-V staining, the Annexin-V-FITC Apoptosis Detection Kit (BMS500FI/300, from eBioscience) was used. Flow cytometry was performed on a BD Biosciences FACSCalibur or BD Biosciences Canto with data analysis using the FlowJo software (Tree Star, Olten, Switzerland).

LN cell suspensions

Skin-draining LNs (brachial, inguinal, and auricular) were teased apart and digested (25 minutes/37 °C) with 0.12 mg ml^{-1} of DNAse I (Roche, Mannheim, Germany) and 0.5 mg ml^{-1} collagenase D (Roche). The resulting cell suspension was used for antibody labeling and subsequent flow cytometric analysis.

Skin cell suspensions

For epidermal cell suspensions, ear and trunk skin were floated on 0.8% trypsin (Merck, Darmstadt, Germany) for 15–45 minutes at 37 °C, respectively (Stoitzner *et al.*, 2010). For digestion of total skin, trunk skin was cut into small pieces and transferred into Hank's medium (w/o $Ca^{2+}Mg^{2+}$, Biochrom AG, Berlin, Germany), supplemented with 0.15 mg ml^{-1} Liberase (Roche) and 0.12 mg ml^{-1} DNAse I (Roche) for 1 hour at 37 °C.

Epidermal sheets

Epidermis was separated from the dermis using 0.1 M ammoniumthiocyanate (Merck). The epidermis was peeled off and fixed in acetone (Sigma-Aldrich, Vienna, Austria) or 4% paraformaldehyde (SAV-LP, Flintsbach, Germany). LCs *in situ* were stained with an antibody for MHC-class II (2G9-FITC, BD Biosciences).

CHS assay

Two-month-old Langerin-$p14_{del}$ and littermate control mice were sensitized (day 0) by applying 100 μl of 1% (high dose) or 0.5% (low dose) TNCB (dissolved in acetone: olive oil vehicle, at a ratio of 4:1) onto the shaved abdominal skin. Five days later, the sensitized mice were challenged by applying 25 μl of either 0.5% (high dose) or 0.25% (low dose) TNCB onto the dorsal side of the right ear. As a control, the left ear of the mice was treated with the vehicle only. After 24 hours, the ear thickness was measured. The actual ear swelling was calculated by subtracting ear thickness of the vehicle-treated ear from the TNCB-treated ear.

BMDCs/TGFβ1 blocking assay

Bone marrow was isolated from the hind limbs (femur and tibia). Erythrocytes were removed using an ammonium chloride buffer. Unfractionated bone marrow cells were cultured in complete medium (RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-

glutamine, and 50 μg ml^{-1} gentamycin (all from PAA, GE Healthcare, Vienna, Austria) containing 20 ng ml^{-1} of GM-CSF (14-8332-62, eBioscience) for 8 days. LCL-BMDCs were obtained by culturing unfractionated bone marrow in complete medium supplemented with 20 ng ml^{-1} GM-CSF and 5 ng ml^{-1} human TGFβ1 (100-21C, Pepro-Tech, Hamburg, Germany) for 8 days (Valladeau *et al.*, 2002).

Statistics

Data were analyzed with unpaired Student's *T*-test or one- or two2-way analysis of variance with post-hoc test (Bonferroni or Tukey's test). *P*-values <0.05 were considered significant (*), values <0.01 were considered very significant (**), and values <0.001 were considered highly significant (***). Statistics were performed using PRISM 5.0 (Graphpad Software, La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMDCs	bone marrow–derived dendritic cells
CHS	contact hypersensitivity
DCs	dendritic cells
ERK	extracellular signal-regulated kinase
LAMTOR	late endosomal/lysosomal adaptor and MAPK and mTOR activator/ regulator complex
LCL-BMDCs	Langerhans cell-like bone marrow derived dendritic cells
LCs	Langerhans cells
LNs	lymph nodes
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mTOR	mammalian target of rapamycin
TGFβ1	transforming growth factor β1
TNCB	trinitrochlorobenzene

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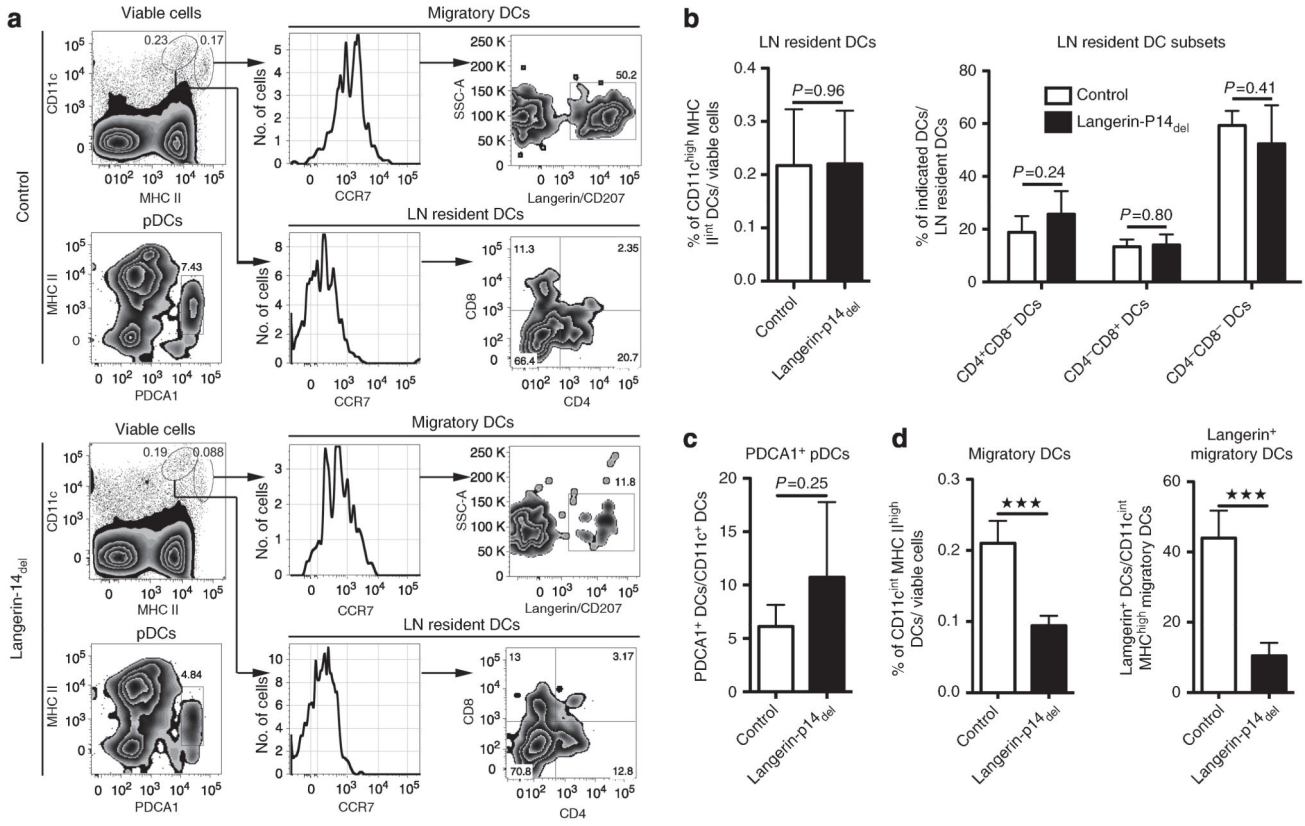

Figure 1. Langerin-specific deletion of p14 leads to loss of Langerin$^+$ migratory dendritic cells (DCs) in the skin-draining lymph nodes (LNs) of adult mice

(**a**) FACS analysis of skin-draining LNs of adult control and Langerin-p14$_{del}$ mice for various DC subsets. LN-resident (CD11c^{high}MHC-class II^{int}DCs) and migratory DCs (CD11c^{int}MHC-class II^{high}) were analyzed by pregating on viable cells and analysis of the chemokine receptor CCR7. Plasmacytoid DCs were characterized as MHC-class II^{low}PDCA1$^+$ DCs among CD11c$^+$ pregated viable cells. One representative experiment is shown in **a**. Combined data for percentages of LN-resident DCs, pDCs, and migratory DCs from four individually analyzed mice per genotype are analyzed in **b, c,** and **d**. Mean±SD is shown, ***P<0.001. MHC, major histocompatibility complex.

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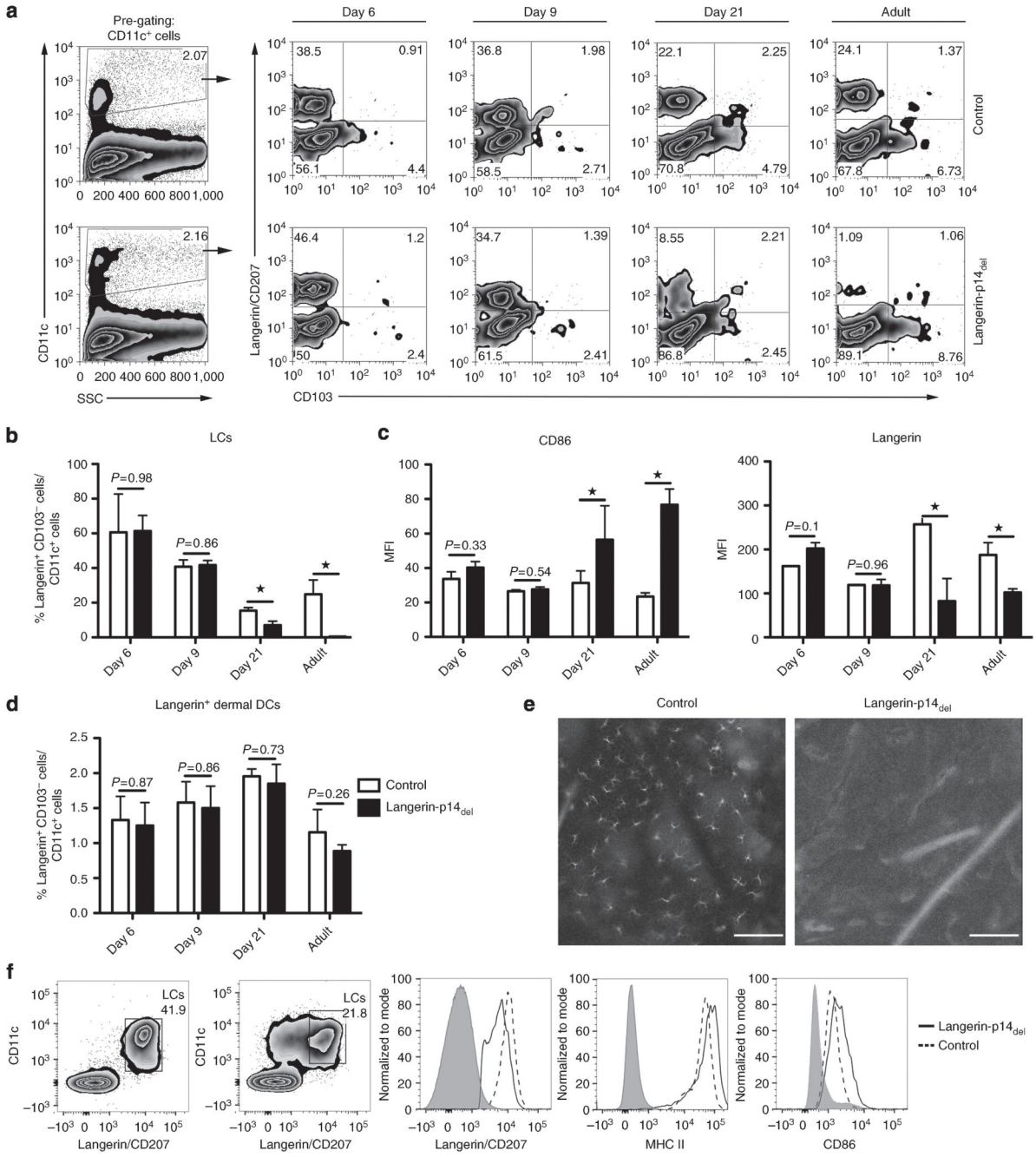

Figure 2. LC numbers decline 3 weeks after birth in Langerin-p14$_{del}$ mice

(**a, b**) FACS analysis of newborn and adult whole skin obtained from Langerin-p14$_{del}$ and control mice for the presence of CD11c^{+}Langerin^{+}CD103^{-} LCs at defined time points after birth. (**c**) Expression of CD86 and Langerin by Langerin^{+}CD103^{-} LCs is shown as mean fluorescence intensity (MFI). (**a, d**) FACS analysis of newborn and adult skin obtained from Langerin-p14$_{del}$ and control mice for the presence of dermal CD11c^{+}Langerin^{+}CD103^{+} DCs at defined time points. One representative experiment is shown in **a**; combined data from six mice per genotype and time point in **b, c**, and **d**. Mean±SD is shown, *P<0.05. (**e**)

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Epidermis from adult control and Langerin-p14$_{del}$ mice was stained for major histocompatibility complex (MHC)-class II; scale bar = 100 μm (**f**) FACS analysis of epidermal cell suspension, derived from 21-day-old Langerin-p14$_{del}$ (second density plot) and control mice (first density plot), for the maturational phenotype of LCs. LCs were identified by the expression of CD11c and Langerin. One representative experiment out of two is shown in **f**.

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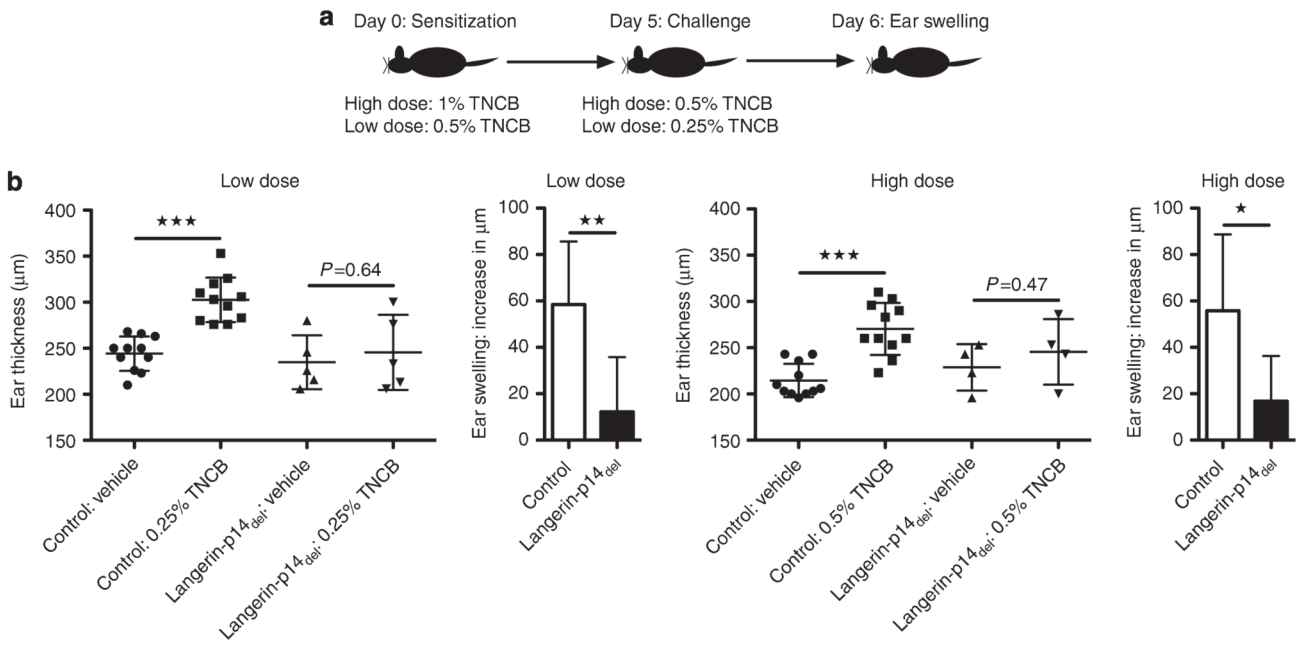

Figure 3. Loss of Langerhans cells (LCs) reduces contact hypersensitivity (CHS) response to trinitrochlorobenzene (TNCB)

(**a**) Scheme for experimental setup. (**b**) Measurement of the ear thickness and the calculation of the actual ear swelling of Langerin-p14$_{del}$ and control mice, treated either with low-dose (0.25%, left side) or high-dose (0.5%, right side) TNCB. Summary of three experiments is shown; mean±SD is shown; *P<0.05, **P<0.01, and ***P<0.001.

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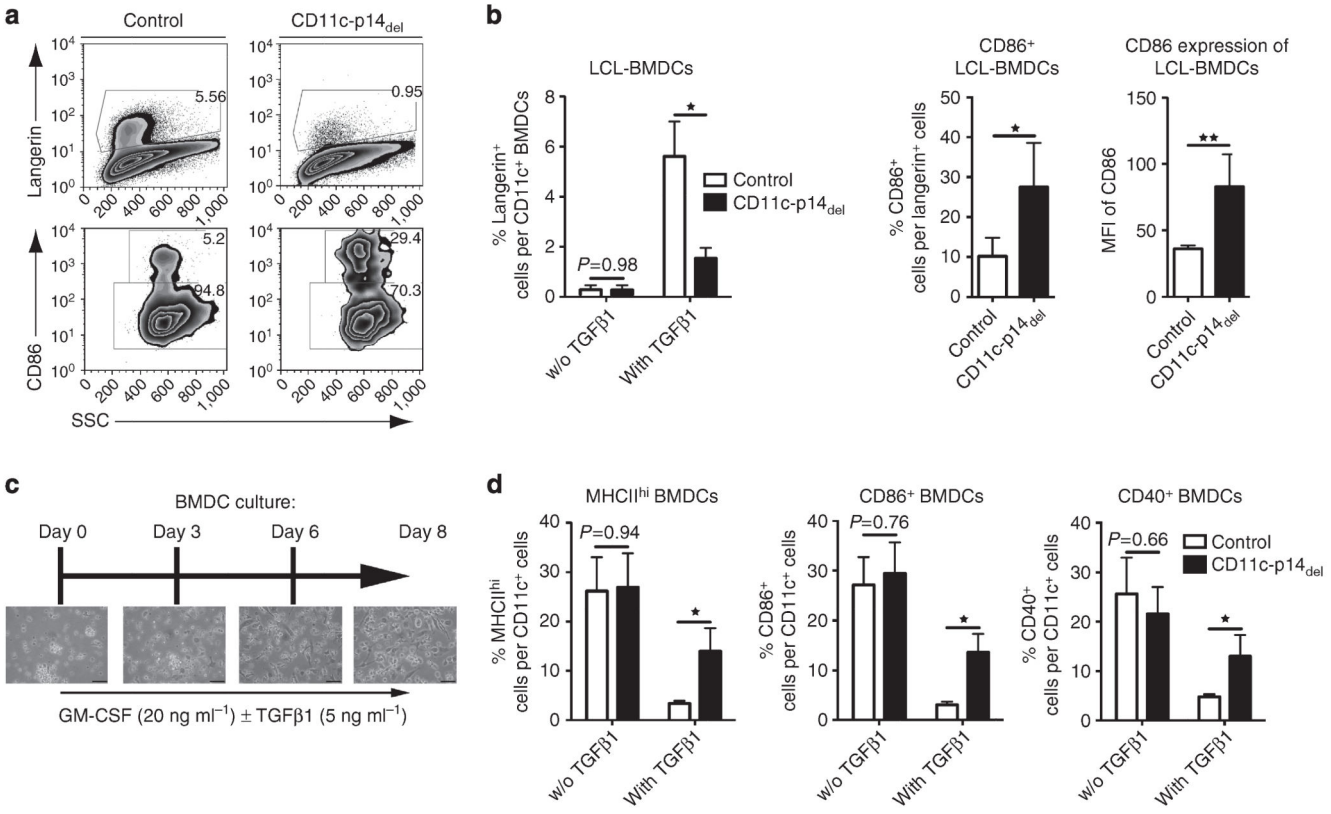

Figure 4. CD11c-specific deletion of p14 impairs responsiveness of bone marrow–derived dendritic cells (BMDCs) toward transforming growth factor β1 (TGFβ1)

(**a, b**) FACS analysis of Langerin$^+$ Langerhans cell (LC)-like BMDCs (LCL-BMDCs) cultured in the presence of GM-CSF±TGFβ1 for 8 days. Viable CD11c$^+$ BMDCs were analyzed for Langerin expression. Maturation was determined by CD86 expression on LCL-BMDCs. One representative experiment for TGFβ-supplemented cultures in **a**; combined data from at least three individually analyzed mice per genotype in **b**. Mean±SD is shown. (**c, d**) FACS analysis of total CD11c$^+$ BMDCs cultured in the presence of GM-CSF±TGFβ1 for 8 days. Maturation was determined by the expression of major histocompatibility complex (MHC)-class II, CD86, and CD40 on CD11c$^+$BMDCs. BMDC culture scheme in **c**, scale bar = 50 μm; combined data from three individually analyzed mice per genotype in **d**. Mean±SD is shown, **P*<0.05, ***P*<0.01.

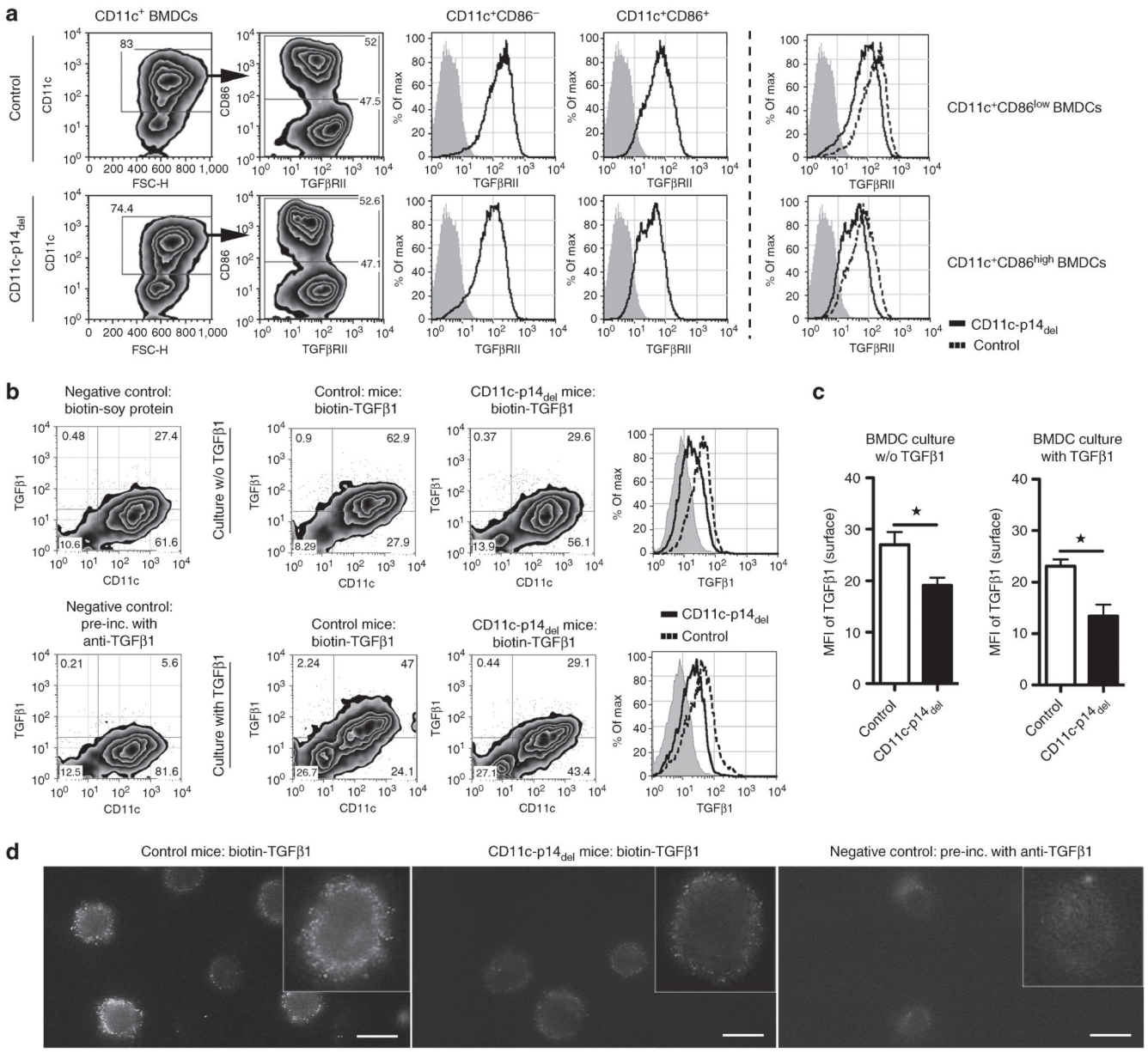

Figure 5. Ablation of p14 reduces the expression of transforming growth factor βRII (TGFβRII) and the surface binding of TGFβ1 to bone marrow–derived dendritic cells (BMDCs)

(**a**) Analysis of $CD11c^+$ BMDCs, obtained from CD11c-$p14_{del}$ and control mice, on day 8 of culture for the expression of the TGFβRII. BMDCs were analyzed by discriminating between $CD11c^+CD86^-$ and $CD11c^+CD86^+$ BMDCs. (**b, c**) Binding of biotinylated TGFβ1 to day 8 $CD11c^+$ BMDCs cultured without (upper row) or with TGFβ1 (lower row); isotype, gray filled; control mice, dotted line; CD11c-$p14_{del}$ mice, black line. Biotinylated soy protein or preincubation with an anti-TGFβ1 blocking antibody was used for negative controls. One representative experiment of each BMDC culture condition in **a** and **b**, combined data from four individually analyzed mice per genotype and culture condition in **c**. Mean±SD is shown. (**d**) Microscopic analysis of (green fluorescent) TGFβ1 surface

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binding on day 8 CD11c-p14$_{del}$ and control BMDCs. Preincubation with an anti-TGFβ1 blocking antibody was used as negative control. Scale bar = 10 μm. *P<0.05.

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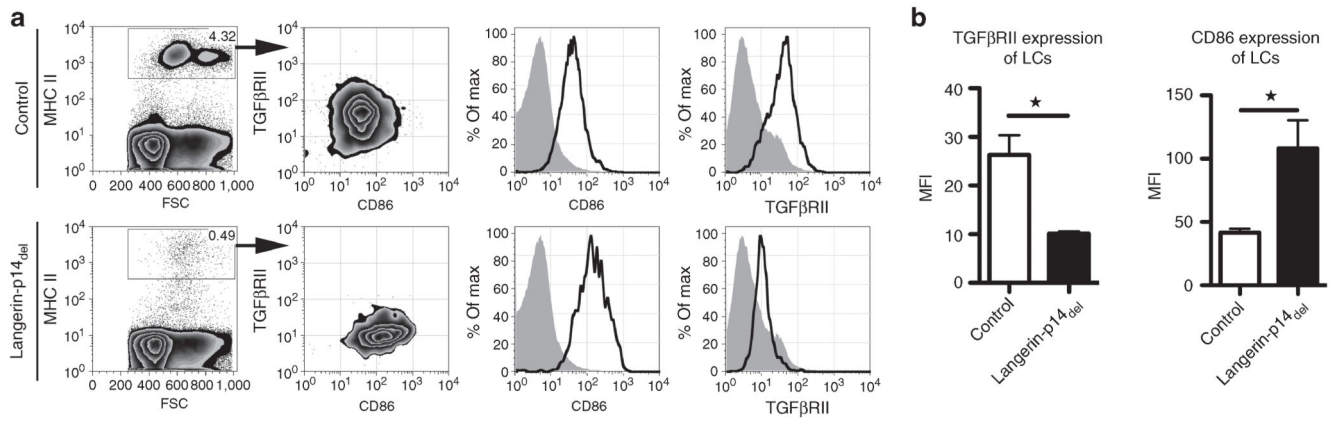

Figure 6. TGFβRII is downregulated on Langerhans cells (LCs) upon p14 deletion

(**a, b**) FACS analysis of LCs isolated from the epidermis of 21-day-old Langerin-p14$_{del}$ and control mice for the expression of TGFβRII. Histogram shows the expression of CD86 and TGFβRII on major histocompatibility complex (MHC)-class II^{+}LCs. One representative experiment in **a**; combined data from three individually analyzed mice per genotype in **b**. Mean±SD is shown, **P*<0.05.

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